In Vitro Metabolism of PCDD and PCB Congeners using Rat Hepatic Microsomes induced by 3-Methylcholanthrene

Nigel J. Bunce and Keke Hu, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.

Abstract

Liver microsomes from untreated and 3-methylcholanthrene-induced immature male Sprague-Dawley rats were used to study the rates of Phase I oxidation of PCDDs and PCBs. The induced microsomes showed > 50 × overexpression of CYP 1A1 and ~ 2 × that of CYP 1A2 compared with controls. The chlorinated substrates were oxidized in the presence of $NADPH/NADP^+$ at pH 7.4 following pseudo-first order kinetics. Two structure-reactivity relationships could be discerned: (1) for either planar or non-planar congeners the rate of oxidation decreased with increasing chlorination, especially of the lateral positions of PCDDs; (2) for PCB congeners the rate of oxidation decreased as the number of *ortho* chlorine atoms in the molecule increased. Michaelis-Menten kinetics were followed for the only case studied in detail so far, namely 4,4′-dichlorobiphenyl: the apparent value of K_m was 1.33×10^{-4} M.

Introduction

Polycyclic aromatic hydrocarbons and their chlorinated derivatives are both inducing agents and oxidizable substrates for monooxygenase enzymes, notably CYP 1A1 and CYP 1A2. The toxicity of chlorinated aromatic compounds is exacerbated by their persistence *in vivo*, which results from their feeble chemical and biochemical reactivity. The toxic potency of PCDDs and other DLCs correlates broadly with their affinity for the cytosolic Ah receptor, thus providing a link between toxic potency and mechanism of action[1]. However, some DLCs that bind the Ah receptor act as antagonists rather than agonists towards various toxic and biochemical endpoints, *e.g.*, the incidence of cleft palate in mouse pups following maternal exposure during gestation[2,3]. Other species and endpoints that have been examined[4,5] have shown additive[2,3,5], synergistic[2,3], and antagonistic[3,6] effects caused by mixtures of TCDD and various other DLCs. We have proposed that the possibility of synergism or antagonism among DLCs depends on the extent of Ah receptor occupancy[7], with antagonism being a possible outcome only at high total receptor occupancy and synergism only at low total receptor occupancy. Other substances have significant binding affinity for the Ah receptor, but no toxic consequences, apparently because the xenobiotic is rapidly metabolized; indole-3-carbinol is an example[8]. Thus the toxic potency of a DLC congener depends upon at least three factors: its inherent potency, the presence and amounts of other agonists and antagonists, and its rate of metabolism. In this work we have studied the *in vitro* oxidation of a range of DLCs, in order to understand the structure-activity relationships underlying this process and the way in which metabolism modifies their toxic potency.

Experimental Methods

Chemicals: 2,3,7,8- and 1,2,3,4-TCDD were purchased from Cambridge Isotope, MA; 1,2,4,7,8-PeCDD, 3,4,4',5-TCB, 2,3,4,4',5-PeCB- and 3,3',4,4',5-PeCB, and all HxCBs were purchased from AccuStandard Inc., New Haven, CT; 2,4'5-TriCB was obtained from Ultra Scientific, Hope, RI; 1,2,3,7,8-PeCDD, 2,2'-DCB, 4,4'-DCB- and 2,2',5,5'-TCB were synthesized in our laboratory.

Animals: Male Sprague Dawley rats (about 100 g) were caged in two groups of six. Treated animals were injected *i.p.* with 3-methylcholanthrene (100 μmol/kg; 26.8 mg/kg) in corn oil on Days 1, 2, 3 and 4 and sacrificed on Day 6. Animals were fasted for 24 h before sacrifice to lower liver glycogen levels. Controls were similarly injected with corn oil.

Microsomal Preparations: Animals were sacrificed by cervical dislocation following anesthesia with CO_2, and the livers were immediatley perfused *in situ* by the hepatic portal vein with ice-cold HEGD buffer, containing HEPES (1 mM), EDTA (1 mM), glycerol (10%, v/v) and DTE (1 mM) at pH 7.6. They were then removed, rinsed once with 15 ml of fresh ice-cold HEGD buffer, weighed, and finely minced. The minced livers were rinsed again with buffer and homogenized in ice-cold buffer using a Teflon-glass Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 10,000 *rpm* for 20 min at 4 °C, followed by centrifugation at 38,000 *rpm* for 68 min at 4 °C. The microsomal pellet was washed, resuspended, and centrifugated again. The pellet was resuspended in the HEGD buffer and stored at -70 °C in small aliquots. Protein content was determined by both Bradford[9] and modified Lowry[10] assays. CYP 1A1 and 1A2 activities were determined by the EROD assay[11,12] and the acetanilide hydroxylase (ACOH) assay[13], respectively.

Kinetic experiments: The rates of *in vitro* metabolism of PCDDs and PCBs were determined by their incubation with 50 μL of liver microsomes at 37 °C in the presence of: Tris-HCl buffer (50 mM, pH 7.4), $MgSO_4$ (5 mM), NADH (0.515 mg), $NADP^+$ (0.5 mM), DL-isocitric acid (7.5 mM), isocitrate dehydrogenase (1 unit) and albumin (0.539 mg) in a volume of 0.98 mL. After pre-incubation for 5 min, 0.020 mL of the substrate solution was added to each tube. The reaction in a particular incubation tube was quenched after a convenient time by the addition of 1.0 ml of acetone. After extraction with 2×2 mL and 1×1 mL of hexane, the dried extracts were evaporated under N_2, and the residue redissolved in HPLC grade methanol (0.2 mL).

HPLC Methods: λ_{max} values of all substrates were determined (Shimadzu Model UV160U UV-Vis spectrophotometer) prior to HPLC analysis. The HPLC system included Waters U6K universal liquid chromatograph injector, Waters 600E system controller, Waters 490E programmable multiwavelength detector and Baseline 815 HPLC software. Substrates and their metabolites were separated on a Waters μBondpak C_{18} 3.9 mm × 300 mm column using a 90% or 100% methanol (flow rate in the range 1.6-2 mL/min, depending on the congener). Calibration curves of peak area *vs.* concentration were prepared for all substrates.

Results and Discussion

The initial characterization of the microsomal preparations showed a substantial difference between the Bradford and modified Lowry methods (Table 1), but the methods were consistent between treated animals and controls. CYP 1A2 activity as estimated by acetanilide hydroxylase

assay was high even in the control microsomes, but whereas 3-MC induced CYP 1A1 activity by about 90-fold, it only increased the ACOH activity two-fold.

Table 1. Results of Microsome Preparation

rat group	control	3-MC-induced
protein content (Bradford, mg/ml)	13.6 ± 0.8	17.0 ± 1.7
protein content (Lowry, mg/ml)	32.6 ± 6.5	40.1 ± 10.3
P450 1A1 activity (EROD) (nmol/min/mg microsomes)	0.058	5.07
P450 1A2 activity (ACOH) (nmol/min/mg microsomes)	4.46	11.35

$$[E]\ (\text{enzyme conc}) = \frac{\text{EROD activity} \times [\text{protein}],\ \text{Bradford} \times \text{volume of enzyme}}{\text{enzyme dilution factor} \times \text{volume os solution incubated}}$$

Table 1 indicated that these oxidations might involve catalysis by CYP 1A1, CYP 1A2, or both. Comparison of kinetic data from the control and induced microsomes showed that CYP 1A1 is chiefly responsible for catalyzing the oxidations of 4,4´-DCB and 1-CDD: Table 2.

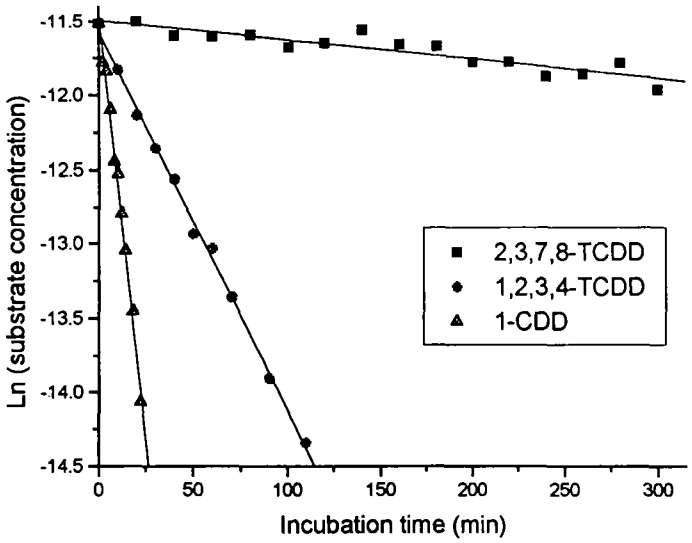

Figure 1. Kinetic plots for 2,3,7,8-TCDD, 1,2,3,4-TCDD and 1-CDD, [E] = 0.215 nmol /ml/min

At 109 × dilution, the 3-MC-induced microsomes had the same EROD activity as the control microsomes. For both 1-CDD and 4,4´-DCB the oxidation was first order in enzyme (the expected rate ratios for 20: 87: 109 × dilutions are 5.5: 1.3: 1.0; the experimental values

were 4.5: 1.2 : 1.0 for 1-CDD and 6.3: 1.5: 1.0 for 4,4´-DCB. In the case of 1-CDD the rate of oxidation by induced microsomes at 109 × was the same (± 7%) as that of control microsomes, showing that essentially all the activity towards this substrate originates with CYP 1A1. For 4,4´-DCB the rate constants differed by a factor of 2, even after taking into consideration the higher substrate concentration used with the control microsomes. However, the fact that the value for controls was lower than expected, not higher, again indicates that the enzymic activity was predominantly contributed by CYP 1A1.

Table 2A. Kinetic Results for 1-CDD

Microsomes	Control*	Induced	Induced	Induced
Dilution factor	1 ×	20 ×	87 ×	109 ×
[E] (nmol/min/mL)	0.0394	0.215	0.0493	0.0395
k, min^{-1}	0.0234	0.114	0.0299	0.0251
k´ (mL/nmol) = k/[E]	0.593	0.530	0.606	0.636

* Initial [1-CDD] was 2.0×10^{-4} M in this experiment; all other experiments used 1.0×10^{-4}M.

Table 2B. Kinetic Results for 4,4'-DiCB

Microsomes	Control	Induced	Induced	Induced
Dilution factor	1 ×	20 ×	87 ×	109 ×
[E] (nmol/min/mL)	0.0394	0.215	0.0493	0.0395
k, min^{-1}	0.0030	0.0864	0.0212	0.0136
k´ (ml/nmol)	0.076	0.402	0.431	0.345

Table 2C. Kinetic Results for 1,2,3,4-TCDD, 2,3,7,8-TCDD and 1,2,4,7,8-PeCDD

microsomes	1,2,3,4-TCDD		2,3,7,8-TCDD		1,2,4,7,8-PeCDD	
	Control	Induced	Control	Induced	Control	Induced
Dilution factor	1 ×	20 ×	1 ×	20 ×	1 ×	20 ×
[E] (nmol/min/ml)	0.0394	0.215	0.0394	0.215	0.0394	0.215
k (min^{-1})	0.0022	0.0255	0.0010	0.0013	0.0008	0.0012
k'(ml/nmol)	0.055	0.119	0.025	0.006	0.019	0.006

The two TCDD congeners and two PeCDD congeners gave somewhat different results. 1,2,3,4-TCDD behaved very similarly to 4,4´-DCB; their reactivities as judged by half-lives

were less than a factor of two apart, and in each case the induced enzyme was 2 × more active on the basis of equal EROD activities. 2,3,7,8-TCDD and 1,2,4,7,8-PeCDD were oxidized much more slowly than 1-CDD; each of them was 5 × less readily oxidized by the induced than by the control microsomes on the basis of equal EROD activity, suggesting the possible involvement of CYP 1A2 in these cases. 1,2,3,7,8-PeCDD was not oxidized at a detectable rate in the presence of either induced or control microsomes.

Table 3 summarizes the rate constants for oxidations of PCBs that we have measured so far, in each case using induced microsomes at the 20-fold dilution. The data are arranged in four groups (CDDs, coplanar PCBs, PCBs with one *ortho* chlorine, PCBs with > 1 *ortho* chlorine. The following structure-reactivity trends may be noted qualitatively. Within each group, oxidizability decreases progressively with increased chlorine substitution.

Table 3. Kinetic Results for PCDDs and PCBs at [E] = 0.0215 nmol/min/mg induced microsomes

Substrate	k′, mL/nmol	Substrate	k′, mL/nmol	Substrate	k′, ML/nmol
4,4'-DiCB	0.402	2,4',5-TriCB	0.109	2,2'-DiCB	0.000
3,4,4',5-TCB	0.005	2,3,4,4',5-PeCB	0.000	2,2',5,5'-TCB	0.001
3,3',4,4',5-PeCB	0.001	2,3,3',4,4',5-HxCB	0.000	2,2',4,4',5,5'-HCB	0.002
3,3',4,4',5,5'-HxCB	0.000			2,2',4,4',6,6'-HxCB	0.000

The oxidation of 4,4′-DCB using induced microsomes followed Michaelis-Menten kinetics (Figure 2). The Lineweaver-Burke plot was linear, $r^2 = 0.9715$, and afforded $K_m = 1.33 \times 10^{-4}$ M, indicating a fairly strong interaction between the substrate and the enzyme's active site. Further experiments are underway to extend this approach to other substrates.

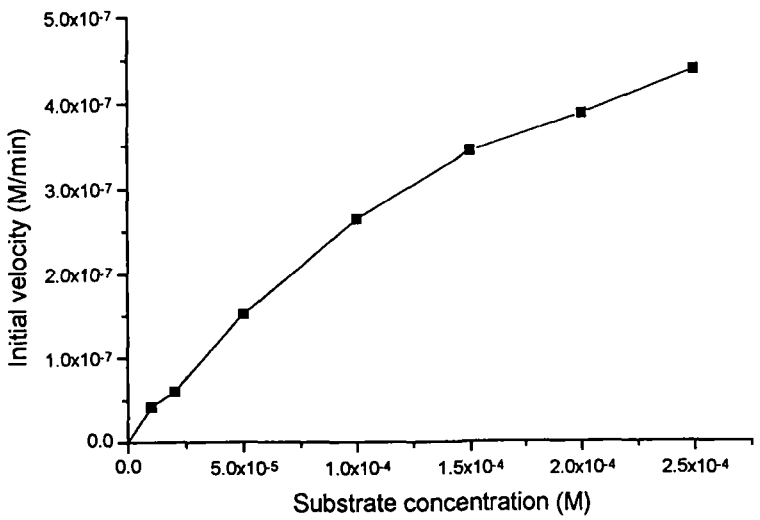

Figure 2. Michaelis-Menten kinetics for 4,4'-diCB, [E] = 0.0108 nmol/ml/min

Acknowledgments

Financial support for this work was provided by the Natural Sciences and Engineering Research Council of Canada.

Literature Cited

1. Safe, S.H., Ann. Rev. Pharmacol. Toxicol., **1986**, 26, 371-399.

2. Birnbaum, L. S.; Weber, H.; Harris, M. W.; Lamb IV, J. C.; McKinney, J. D. *Tox. Appl. Pharm.* **1985**, 77, 292-302.

3. Biegel, L.; Harris, M.; Davis, D.; Rosengren, R.; Safe, L.; Safe, S. *Tox. Appl. Pharm.* **1989**, 561-571.

4. Santostefano, M.; Liu, H,; Wang, X.; Chaloupka, K.; Safe, S. *Chem. Res. Toxicol.* **1994**, 7, 544-550.

5. van Birgelen, A. P. J. M.; Fase, K. M.; van der Kolk, J.; Poiger, H.; Brouwer, A.; Seinen, W.; van den Berg, M. *Environ. Health Pers.* **1996**, 104, 550-557.

6. Merchant, M.; Morrison, V.; Santostefano, M.; Safe, S. *Arch. Biochem. Biophys.* **1992**, 298, 389-394.

7. Brown, M. M.; Schneider, U. A.; Petrulis, J. R.; Bunce, N. J. *Tox. Appl. Pharm.* **1994**, 129, 243-251.

8. Chen, I; Harper, N.; Safe, S., *Organohalogen Comp.*, **1995**, 25, 57-60.

9. Bradford, M.M. *Anal. Biochem.* **1976**, 72, 248-254.

10. Peterson, G.L. *Anal. Biochem.* **1977**, 3, 346-356

11. Burke, M.D. and Mayer, R.T. *Drug Metab. Dispos.* **1974**, 2, 583-588.

12. Lake, B.G. and Paine, A.J., *Xenobiotica* **1983**, 13, 725-730

13. Liu, G., Gelboin, H.V. and Myers, M.J. *Arch. Biochem. Biophys.* **1991**, 284, 400-406